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Renee Levings
University of Wollongong

Diane Lightfoot
University of Melbourne

Sally R. Partridge
Macquarie University

Ruth M. Hall
University of Sydney

Steven P. Djordjevic
Elizabeth Macarthur Agricultural Institute

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Abstract

The global dissemination of the multiply-antibiotic-resistant *Salmonella enterica* serovar Typhimurium DT104 clone with the resistance genes located in a class 1 integron, here designated In104, within genomic island SGI1 is a significant public health issue. Here, we have shown that SGI1 and variants of it carrying different combinations of resistance genes are found in several *Salmonella enterica* serovars. These are serovars Cerro, Derby, Dusseldorf, Infantis, Kiambu, and Paratyphi B dT⁺ isolated from human infections and serovar Emek from sewage effluent. Two new variants, SGI1-I and SGI1-J, both of which include the *dfxA1*-orfC cassette array, were identified.

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The Genomic Island SGI1, Containing the Multiple Antibiotic Resistance Region of *Salmonella enterica* Serovar Typhimurium DT104 or Variants of It, Is Widely Distributed in Other *S. enterica* Serovars

Renee S. Levings,^{1,2} Diane Lightfoot,³ Sally R. Partridge,⁴§ Ruth M. Hall,⁵
and Steven P. Djordjevic^{1*}

New South Wales Department of Primary Industries, Elizabeth Macarthur Agricultural Institute, Microbiology and Immunology Section, Camden, New South Wales 2570,¹ Department of Biological Sciences, University of Wollongong, New South Wales 2522,² Microbiological Diagnostic Unit, Public Health Laboratory, Department of Microbiology and Immunology, University of Melbourne, Victoria 3013,³ Department of Biological Sciences, Macquarie University Sydney, New South Wales 2109,⁴ and School of Molecular and Microbial Biosciences, University of Sydney, New South Wales 2006,⁵ Australia

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The global dissemination of the multiply-antibiotic-resistant *Salmonella enterica* serovar Typhimurium DT104 clone with the resistance genes located in a class 1 integron, here designated In104, within genomic island SGI1 is a significant public health issue. Here, we have shown that SGI1 and variants of it carrying different combinations of resistance genes are found in several *Salmonella enterica* serovars. These are serovars Cerro, Derby, Dusseldorf, Infantis, Kiambu, and Paratyphi B dT⁺ isolated from human infections and serovar Emek from sewage effluent. Two new variants, SGI1-I and SGI1-J, both of which include the *dfx1A1*-orfC cassette array, were identified.

Most multiply-antibiotic-resistant (MR) *Salmonella enterica* serovar Typhimurium DT104 isolates exhibit resistance to ampicillin, chloramphenicol, florfenicol, streptomycin, spectinomycin, sulfonamides, and tetracycline but, because screening for spectinomycin and florfenicol resistance is not routine, are often incorrectly described as “pentaresistant.” Multidrug resistance in the majority of DT104 strains is due to the presence of five genes *aadA2*, *sulI*, *floR*, *tetA(G)*, and *blaP1* (variant form *bla*_{PSE-1} or *bla*_{CARB-2}) that confer resistance to streptomycin and spectinomycin, sulfonamides, chloramphenicol and florfenicol, tetracyclines, and β -lactam antibiotics, respectively (4, 5). These isolates represent a single clone that has emerged as a dominant serovar Typhimurium clone globally and has been isolated from humans, most meat-producing animals, exotic birds, and various foods, including meat, dairy products, and salad ingredients (43, 45).

Molecular typing studies have shown that this MR DT104 clone appeared simultaneously in the United Kingdom, Europe, and the United States during the mid-1980s (9). MR DT104 with the same resistance profile (ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline) had been isolated from humans in Asia prior to 1982 (22), but it is not currently known if these are identical to the globally disseminated clone. MR DT104 is now the second most prevalent *Salmonella* serotype isolated from humans in

the United Kingdom, and the incidence of this clone has increased significantly in the United States, South Africa, the United Arab Emirates, Trinidad, the Philippines, the Irish Republic, Canada, Israel, Japan, and most European countries (36, 43, 45). However, MR DT104 has not taken hold in Australia, where it has been detected only infrequently in imported foods and travelers returning from overseas (D. Lightfoot, unpublished observations).

The resistance genes in the major MR DT104 clone all lie within the boundaries of a large (13 kb) class 1 integron with a complex structure, here named In104 (Fig. 1). The sequence of this region (4, 5) has revealed that the integron has a backbone similar to that of In4 (28, 29), but it includes duplications of parts of the integron conserved segments (CS), specifically, part of the *intI1* gene from the 5'-CS and part of the 3'-CS (*qacE Δ 1* and partial *sulI* genes) that normally flank integrated gene cassettes (16, 17). As a consequence, In104 includes two *attI1* sites, into which gene cassettes can be incorporated (8, 16, 17, 35), with the *aadA2* gene cassette in one and the *blaP1* cassette in the other (37, 39). This arrangement produces a characteristic pair of PCR products of 1.0 kb and 1.2 kb using standard primers that amplify the cassette-containing regions, and these regions have often been called InC and InD (37, 39), assuming there are two integrons rather than one. The *floR* and *tetRA(G)* genes lie between the two integron-derived regions (5) together with an open reading frame, orf2, that is potentially part of an unusual type of insertion sequence recently named CR3 (32).

In104 lies within a genomic island known as SGI1 (4) (GenBank accession no. AF261825). It appears to have moved to this location by transposition because there is a 5-bp direct duplication flanking the 25-bp inverted repeats (IRs) of the

* Corresponding author. Mailing address: New South Wales Department of Primary Industries, Private Mail Bag 8, Camden, New South Wales 2570, Australia. Phone: 61246-406426. Fax: 61246-406384. E-mail: steve.djordjevic@agric.nsw.gov.au.

§ Present address: Centre for Infectious Diseases and Microbiology, Westmead Hospital, University of Sydney, New South Wales 2145, Australia.

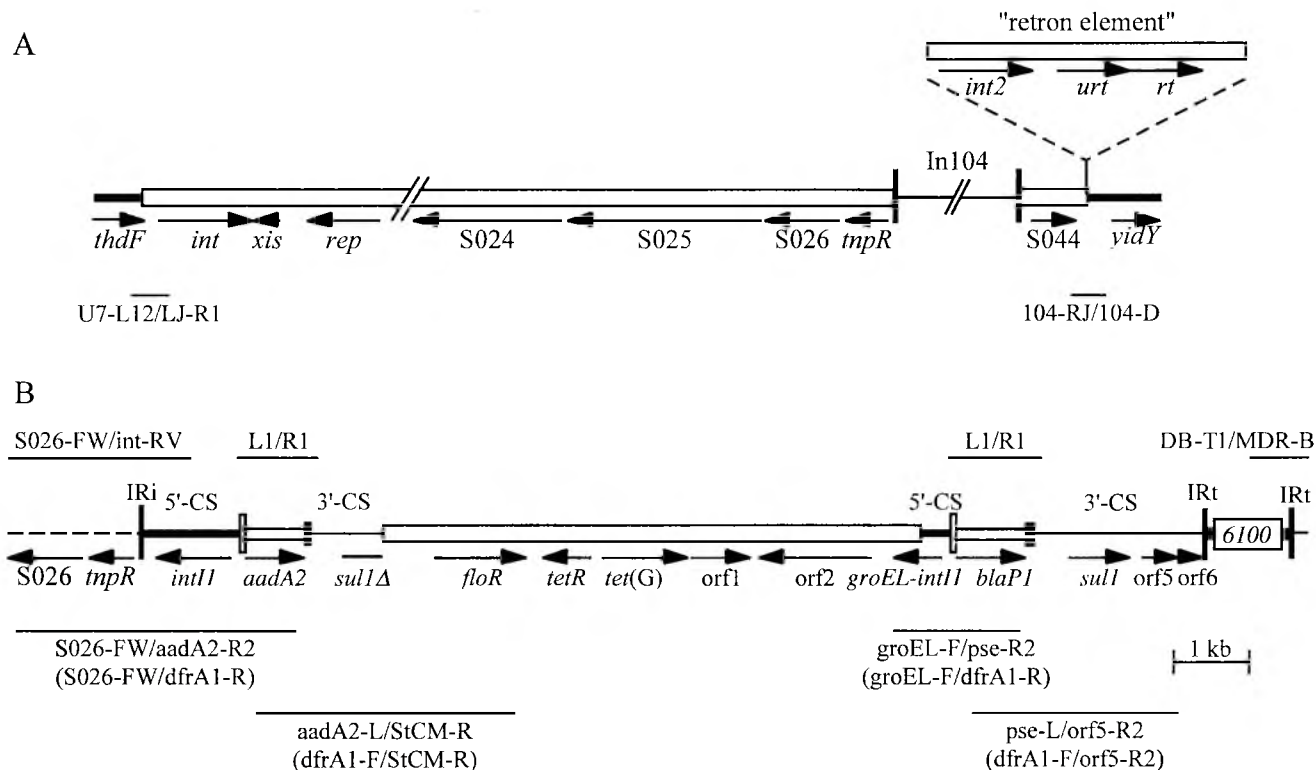


FIG. 1. Structure of SGI1 regions. (A) The SGI1 region of serovar Typhimurium DT104 (from GenBank accession no. AF261825) is drawn to scale except for In104, which is to scale in B. The SGI1 backbone and the retron element (shown above) are represented by open boxes, and the chromosomal and In104 regions as lines of different thicknesses. Vertical bars indicate the IR bounding In104. Fragments amplified by PCR are shown as thin lines below, with the primer pairs used (Table 1) indicated underneath. (B) In104 region. Dashed lines represent the surrounding SGI1 backbone and narrow vertical bars represent the IRs (IRi and IRt). The 5'-CS, 3'-CS, and *int1* regions of class 1 integrons are indicated by lines of different thicknesses, with *attI1* as a tall open box and gene cassettes as open boxes with a black bar at one end, indicating the 59-be. IS6100 and the central, non-integron-derived region are open boxes. Arrows indicate the position and orientation of genes and ORFs. Primer pairs used to detect the boundaries of In104 or to determine the size of the cassette array and the location of cassettes are indicated above or below.

integron and it is located in a presumptive *res* site adjacent to a resolvase gene (*tmpR*) in the SGI1 backbone, which is where class 1 integrons are most commonly found (18, 29). SGI1 has been completely sequenced and is a 43-kb genomic island located in the chromosome between the *thdF* and *yidY* genes (4). Based on the fact that an *int* gene encoding a site-specific recombinase belonging to the tyrosine recombinase family was found within SGI1, at the left-hand end adjacent to the *thdF* gene, SGI1 is likely to have been integrated into the chromosome by site-specific recombination (4).

A second integrated element that also includes an *int* gene and that has been called a retron-phage was found between SGI1 and the *yidY* gene in DT104 strains (4) but is not usually present in other *S. enterica* serovars (3, 10, 12, 13, 24). SGI1 was subsequently found in serovar Typhimurium strains typed as DT1, DT12, DT120, and U302 (3, 6, 19), but it has been shown that the DT12 and DT120 strains probably arose by changes in the phage susceptibility of the SGI1-containing DT104 strain (19). However, SGI1 has also been found in other serovars with diverse sources; Agona isolated from poultry in Belgium (3, 10), Paratyphi B dT⁺ (formerly biovar Java) from tropical fish in Singapore (24), Albany from food fish in Thailand (12), Meleagridis from an unspecified animal in the United States (14), and Newport, isolated from a human (13), confirming that it is not confined to the serovar Typhimurium

or to DT104 strains and suggesting that it may be able to spread by horizontal transfer.

Variations in the structure of the integron, all of which could have arisen by homologous recombination, have also been identified (3, 6, 10, 12, 13). One type of variation involves loss of some of the resistance genes, which can occur via homologous recombination between one or other pair of duplicated regions, namely the 5'-CS or 3'-CS, causing loss of either *aadA2*, *floR*, and *tet(G)* or *floR*, *tet(G)*, and *blaP1* (3, 6). Other variations involve the exchange of one or both of the cassette arrays to introduce different gene cassettes (10, 12, 13), which can also occur via homologous recombination (30, 31). A further source of variation (3) is the acquisition of the *dfrA10* trimethoprim resistance gene (27) together with the sequences that flank it in a different type of complex class 1 integron that has a duplicated 3'-CS and a potential insertion sequence, CR1, that is related to CR3 (3, 32). Acquisition of this segment introduces an additional copy of the *sul1* gene and has been shown experimentally to occur via homologous recombination (30).

Here we have screened a set of resistant and MR *S. enterica* isolates belonging to 20 different serovars and derived from various sources for the presence of SGI1. All strains were isolated in Australia and originated from human infections, acquired either locally and overseas, or from sick animals or environmental sources in Australia. The structure of the class

TABLE 1. Primers

Primer	Sequence (5'-3')	Location	Nucleotide positions	Accession no.	Reference ^a
U7-L12	ACA CCT TGA GCA GGG CAA AG	<i>thdF</i>	1-20	AF261825.2	4
LJ-R1	AGT TCT AAA GGT TCG TAG TCG	<i>int</i>	500-480	AF261825.2	4
SGI19-F3	TTC TTC AAT GCG CGC TTT GG	S023	19009-19028	AF261825.2	This study
S026-FW	TCG GGT AAT CTC AGC AGA GC	S026	25021-25040	AF261825.2	6
int-RV	GGG CAT GGT GGC TGA AGG ACC	<i>intII</i>	27266-27246	AF261825.2	6
L1	GGC ATC CAA GCA GCA AGC	5'-CS	27892-27909, 37164-37181	AF261825.2	21
R1	AAG CAG ACT TGA CCT GAT	3'-CS	28900-28883, 38360-38343	AF261825.2	21
aadA2-L	TGT TGG TTA CTG TGG CCG	<i>aadA2</i>	28146-28163	AF261825.2	26
aadA2-R2	TGC TTA GCT TCA AGT AAG ACG	<i>aadA2</i>	28683-28663	AF261825.2	3
QS-1	ATG AAA GGC TGG CTT TTT CTT G	<i>qacEΔI</i>	28953-28974, 38413-38424	AF261825.2	4
sul1-F	GTG ACG GTG TTC GGC ATT CT	<i>sul1</i>	29297-29316, 35757-35776	AF261825.2	20
QS-2	TGA GTG CAT AAC CAC CAC CC	<i>sul1</i>	29674-29655, 31934-31915	AF261825.2	4
MDR-7	AAC CGT GCA TCT ATC GAG C	— ^b	30094-30112	AF261825.2	3
SiCM-L	CAC GTT GAG CCT CTA TAT GG	<i>floR</i>	30640-30659	AF261825.2	3
SiCM-R	ATG CAG AAG TAG AAC GCG AC	<i>floR</i>	31527-31508	AF261825.2	3
F6	TTG GAA ACA GAC GGC ATG G	<i>tetR(G)</i>	31995-31977	AF261825.2	3
tetG-L	CAG CTT TCG GAT TCT TAC GG	<i>tetA(G)</i>	32819-32838	AF261825.2	26
tetG-R	GAT TGG TGA GGC TCG TTA GC	<i>tetA(G)</i>	33662-33643	AF261825.2	26
orf1-F	CGC CGA ATA TCT CAA CTT CCG	orf1	33927-33947	AF261825.2	This study
orf1-R	CCG CGA TGC CGA AAT CCC	orf1	34581-34564	AF261825.2	This study
orf2-F2	ATG CAG TGA GAA GCC GC	orf2	35291-35307	AF261825.2	This study
orf2-R2	TAC TCG AGC ACG GCT TC	orf2	36024-36008	AF261825.2	This study
groEL-F	ATG CCG CCC ATA CCG CCA GC	<i>groEL</i>	36490-36509	AF261825.2	This study
pse-L	AAT GGC AAT CAG CGC TTC CC	<i>blaP1</i>	37495-37514	AF261825.2	26
pse-R2	ACA ATC GCA TCA TTT CGC TC	<i>blaP1</i>	38147-38128	AF261825.2	3
sul1-R	TTT ACA GGA AGG CCA ACG GT	<i>sul1</i>	39424-39405	AF261825.2	20
orf5-F	AGG TTG TGC GGC TGA TGC	orf5	39776-39793	AF261825.2	This study
orf5-R2	CGA GTT CTA GGC GTT CTG C	orf5	40213-40195	AF261825.2	This study
orf6-R	ACT ATC TTC GGC CTT CAC ACG	orf6	40508-40488	AF261825.2	This study
DB-T1	TGC CAC GCT CAA TAC CGA C	IS6100	41120-41138	AF261825.2	3
IS6100-Rv2	AAT GGT GGT TGA GCA TGC C	IS6100	41475-41457	AF261825.2	This study
MDR-B ^c	GAA TCC GAC AGC CAA CGT TCC	S044	41905-41884	AF261825.2	3
104-RJ	TGA CGA GCT GAA GCG AAT TG	S044	42373-42392	AF261825.2	3
C9-L2	AGC AAG TGT GCG TAA TTT GG	Retron element	42868-42887	AF261825.2	3
104-D	ACC AGG GCA AAA CTA CAC AG	<i>yidY</i>	47130-47111	AF261825.2	3
dfrA1-F	CGA AGA ATG GAG TTA TCG G	<i>dfrA1</i>	621-639	X17477.1	This study
dfrA1-R	TTA GAG GCG AAG TCT TGG	<i>dfrA1</i>	1029-1012	X17477.1	This study
orfC-F	CAT TAC GAA GCG AAT GCA CC	orfC	1172-1191	X17477.1	This study
orfC-R	TCT CGA ATC AAG CAG GAA CC	orfC	1534-1515	X17477.1	This study
orf513-F	ATG TCG CTG GCA AGG AAC G	orf513	3521-3539	L06418.4	This study
RH379	ACC AGA GCA TTC GGT AAT CAA G	<i>dfrA10</i>	5530-5551	L06418.4	This study
RH380	GCT TCA GAT AAT AAA CCA ACA CCA CC	<i>dfrA10</i>	5828-5803	L06418.4	This study

^a References are for the primer.

^b MDR-7 targets the sequence preceding *floR*.

^c The sequence from AF261825.2 is GAATCCGACAGCCAACGCTCC.

I integron in isolates containing SGII was analyzed and complete maps were derived using a variety of methods.

MATERIALS AND METHODS

Strain collection. A total of 76 *S. enterica* strains isolated in Australia in 1999 or 2001 and submitted to the Microbiological Diagnostic Unit for typing were examined. Of these, 39 isolates were from humans with infections, with 16 isolates recorded as acquired overseas (recently returned travelers), and the remainder presumed to be acquired locally. The rest of the collection consisted of 36 isolates from Australian animals, pigs (10 isolates), cattle (9 isolates), chickens (9 isolates), ducks (5 isolates), horses (2 isolates), and cats (1 isolate), all presumed to be associated with disease, and one isolate from sewage effluent. A total of 20 different serovars, determined using standard procedures (33), were included in the collection. The collection contained 14 isolates of serovars Typhimurium (six from humans: four bovine; two porcine; two equine), and Hadar (nine human: five duck), seven serovar Sofia (two human: five chicken), six each of serovars Infantis (two human; three chicken; one cat) and Bovismorbificans (one human; five bovine), four Derby (two human; two porcine) and Agona (two human; two porcine), three Kiambu (two human; one chicken), two each of Blockley (human), Havana (one human; one porcine), Ohio (porcine), Paratyphi B dT⁺ (biovar Java) (human), Singapore (human), Stanley (human), single isolates of Cerro, Dusseldorf, Enteritidis, Montevideo (all human), and Seftenberg (porcine), and a single serovar Emek isolate from sewage effluent.

All strains had been shown to be resistant to at least one of the following antibiotics at the concentrations shown: ampicillin (Ap, 32 µg/ml), chloramphenicol (Cm, 10 µg/ml), gentamicin (Gm, 2.5 µg/ml), kanamycin (Km 10 µg/ml), streptomycin (Sm, 25 µg/ml), spectinomycin (Sp, 50 µg/ml), sulfathiazole (Su, 550 µg/ml), tetracycline (Tc, 20 µg/ml), or trimethoprim (Tp, 50 µg/ml) using procedures described previously (1, 2). The collection included a broad range of resistance profiles with six isolates resistant to only one of these antibiotics, 18 isolates resistant to two antibiotics, nine resistant to three antibiotics, seven resistant to four, and 36 resistant to five or more (up to nine) antibiotics. Resistance to nalidixic acid (Na, 50 µg/ml) and ciprofloxacin (Cp, 2 µg/ml) was also recorded in some isolates, mainly from humans. A single SGII-containing DT104 strain, isolated from a human and traced back to an imported food (15), was included for comparison.

PCR. The primers used are listed in Table 1. For initial PCR screening, 5 µl of template prepared by resuspending a single colony in 1 ml of sterile Milli-Q water, heating to 100°C for 5 min, and pelleting debris by centrifugation (13,000 rpm, 10 min) was used. DNA of higher purity was isolated using a standard protocol (38), and 10 to 50 ng was used in all subsequent analyses. For detailed PCR mapping, DNA fragments were amplified in a reaction mixture (final volume of 50 µl) containing 10 mM Tris-HCl (pH 8.3), 10 mM KCl, 2 mM MgCl₂ and 0.2 mM of each deoxynucleoside triphosphate with 20 pmol of each primer and 1 U of *Taq* DNA polymerase. Thermal cycling conditions consisted of an initial denaturation cycle (95°C, 5 min) followed by 35 cycles of denaturation

TABLE 2. Analysis of the boundaries of SGI1 and In104 within SGI1 in multiple-antibiotic-resistant *Salmonella* spp.

Strain	Serovar	Source	Country ^a	Date of isolation (day/mo/yr)	Junctions ^b			
					SGI LJ	SGI RJ	In104 LJ	In104 RJ
SRC 4	Derby	Human	Malaysia	20/05/99	+	+	+	+
SRC 5	Cerro	Human	Thailand	10/09/99	+	+	+	+
SRC 10 ^c	Kiambu	Human	Australia	01/08/99	+	+	+	+
SRC 11 ^c	Kiambu	Human	Australia	01/04/99	+	+	+	+
SRC 19 ^c	Emek	Effluent	Australia	15/09/99	+	+	—	—
SRC 38	Dusseldorf	Human	Malaysia	30/08/01	+	+	+	+
SRC 46	Infantis	Human	Australia	10/02/01	+	+	+	+
SRC 49 ^c	Paratyphi B dT ⁺	Human	Australia	04/09/01	+	+	+	+
SRC 50 ^c	Paratyphi B dT ⁺	Human	Australia	02/05/01	+	+	+	+
DT104-2	Typhimurium	Human	Australia ^f	??/05/01	+	— ^d	+	+

^a Countries listed represent regions recently visited by affected patients.

^b Primer pairs used (3, 4) with product sizes in brackets are SGI1-LJ, U7-L12, and LJ-R1 (0.5 kb); SGI1 RJ, 104-RJ, and 104-D (0.5 kb); In104 LJ, S026FW, and int-RV (2.245 kb); In104 RJ, DB-T1, and MDR-B (0.786 kb).

^c Same species isolates 10 and 11 or 49 and 50 are not known to be epidemiologically linked.

^d DT104 strains with SGI1 possess a retron element that disrupts the boundary spanned by primers 104-RJ/104-D.

^e Primers SGI19(F3) and int-RV were used to link the SGI1 backbone to In104 in SRC19.

^f Isolate linked to the consumption of halva (origin, Turkey) contaminated with DT104 (15).

(95°C, 30 s), annealing at temperatures ranging from 57 to 65°C (30 to 60 s), and extension (72°C, 60 to 120 s), and a final cycle of amplification (72°C, 5 min). Annealing temperatures for all primer pairs shown in Fig. 1 were 60°C except for groEL-F/pse-R2 (65°C), groEL-F/orfC (65°C), and L1/R1 (57°C). Amplified DNA fragments were resolved by electrophoresis using a 1 to 2% (wt/vol) agarose gel, stained with ethidium bromide (5 µg/ml), visualized with UV, and imaged using a GelDoc 1000 image analysis station (Bio-Rad, Hercules, CA).

Hybridization probes for *tet*(G) (amplified with the tetG-L and tetG-R primers) and *orf2* (amplified using *orf2*-F2 and *orf2*-R2 primers) were labeled with digoxigenin using the digoxigenin Easy Hyb kit (Roche Diagnostics Corporation, Indianapolis, Indiana) and the manufacturer's instructions. PCR cycling conditions consisted of a denaturation cycle (94°C, 5 min) followed by 30 cycles of denaturation (94°C, 30 s), annealing at 60°C for *tet*(G) and 64°C for *orf2* (30 s), and extension (72°C, 90 s), and a final extension cycle (72°C, 10 min).

Southern hybridization. DNA was digested with BsaI, resolved by electrophoresis through 0.7% agarose, and transferred to N⁺ nylon membrane (Amersham, Buckinghamshire, United Kingdom) by capillary action as described previously (38). The DNA was cross-linked to the membrane by baking at 80°C for 2 h and exposed to denatured digoxigenin-labeled *tet*(G) and *orf2* probes overnight at 42°C. Membranes were then washed twice with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)/0.1% sodium dodecyl sulfate (SDS) for 15 min at room temperature and twice, more stringently, with 2× SSC/0.1% SDS for 15 min at 68°C. Bands were visualized fluorescently using standard protocols supplied the manufacturer (Roche Diagnostics Corporation). Digoxigenin-labeled molecular mass markers (molecular marker set II, Roche Diagnostics Corporation) were included on all membranes.

DNA sequencing. PCR products were prepared for sequencing using the QIAquick PCR purification kit (QIAGEN, Hilden, Germany) following protocols supplied by the manufacturer. Sequencing was performed using the Big Dye Terminator reaction mix version 3.2 (Applied Biosystems, Foster City, CA). Cycle sequencing reactions (20 µl final volume) contained a PCR amplicon (30 to 90 ng), 3.2 pmol of primer, and 8 µl of reaction mix and consisted of 28 cycles of denaturation (90°C, 30 s), annealing (50°C, 15 s) and extension (60°C, 240 s).

Nucleotide sequence accession number. The sequence of the Emek (SGI1-J) *drfA1*-*orfC* cassette array has been submitted to the GenBank database under accession no. AY963803.

RESULTS

Identification of SGI1-containing strains. All strains were screened by PCR for the presence of SGI1 with or without the retron element using published primer pairs (3) (Table 1) that span the boundaries of SGI1 with the chromosome or with the retron element (Fig. 1A). The MR DT104 control and nine isolates representing seven serovars, Cerro, Derby, Dusseldorf, Emek, Infantis, Kiambu (two isolates), and Paratyphi B dT⁺ (two isolates), contained both ends of SGI1 (Table 2). The

retron element was found only in the DT104 control (data not shown). These strains (Table 2) included 8 of the 39 human isolates in the collection but none originating from animals. The ninth strain was from sewage effluent.

To determine if the integron characteristic of SGI1 and its variants was present, published primer pairs (3, 6) spanning the boundaries of In104 with the SGI1 backbone (Fig. 1B) were used. PCR amplification yielded products of the sizes expected for the integron located in SGI1 in all except the serovar Emek strain, where neither boundary was detected (Table 2). However, using the outward-facing primer internal to the integron together with primers in other SGI1 backbone genes, the left or IRi end of a class 1 integron was found adjacent to part of the S023 gene. The sequence of this fragment revealed that 6,889 bp of SGI1 backbone sequence normally found adjacent to the IRi end of the integron was missing. Primers internal to the IS6100 element at the right end of In104 coupled with right-hand primers in the adjacent chromosomal *uidY* gene did not reveal a junction, suggesting that a large insertion may be present at this end in the serovar Emek strain.

Identification of resistance genes. When the resistance profiles were examined (Table 3), only four of the strains, SRC49 and SRC50 (both serovar Paratyphi B dT⁺ isolates) and SRC10 and SRC11 (both serovar Kiambu isolates), were resistant to all antibiotics in the standard SGI1-containing DT104 set (Ap, Cm and Fl, Sm and Sp, Su, and Tc). Resistance to trimethoprim (Tp) was observed in seven strains and resistance to gentamicin (Gm) in one strain. The nine strains were also screened for the presence of each of the genes and open reading frames (ORFs) found in In104 (see Fig. 1B) using pairs of primers internal to the genes (Table 1). The standard SGI1 resistance phenotype (ApCmFlSmSpSuTc) and resistance gene complement, *aadA2*, *blaP1*, *sul1*, *tetA*(G), and *floR*, consistent with the presence of an integron identical to or closely related to In104, were only present in the two Paratyphi B dT⁺ strains (Table 3). Hence, most of the strains contained a variant of SGI1. Consistent with this conclusion, five strains lacked one or both of the cassette-associated resistance genes *aadA2* (Sm and Sp) and *blaP1* (Ap) (Table 3). In the serovar

TABLE 3. Properties of *Salmonella* spp. containing SGI1 or its variants

Strain	Serovar	Antibiotic resistance profile ^a									Resistance genes						
		Ap	Fl/Cm	Sm/Sp	Su	Tc	Tp	Gm	Cp	Na	<i>blaP1</i>	<i>floR</i>	<i>aadA2</i>	<i>sul1</i>	<i>tetG</i>	<i>dfrA1</i>	<i>dfrA10</i>
SRC 4	Derby		R	R	R	R	R	R									
SRC 5	Cerro	R	R		R	R	R		R	R							
SRC 10	Kiambu	R	R	R	R	R	R										
SRC 11	Kiambu	R	R	R	R	R	R		R	R							
SRC 19	Emek		R		R	R	R		R	R							
SRC 38	Dusseldorf	R	R		R	R	R			R							
SRC 46	Infantis			R	R		R										
SRC 49	Paratyphi B dT ⁺	R	R	R	R	R											
SRC 50	Paratyphi B dT ⁺	R	R	R	R	R											
DT104-2	Typhimurium	R	R	R	R	R											

^a Antibiotics: Ap, ampicillin (32 µg/ml); Cm, chloramphenicol (10 µg/ml); Gm, gentamicin (2.5 µg/ml); Sm, streptomycin (25 µg/ml); Sp, spectinomycin (50 µg/ml); Su, sulfamethoxazole (550 µg/ml); Tc, tetracycline (20 µg/ml); Tp, trimethoprim (50 µg/ml); Cp, ciprofloxacin (2 µg/ml); Na, nalidixic acid (50 µg/ml). Florfenicol (Fl) resistance was tested by streaking isolates onto LB-agar containing 15 µg/ml of florfenicol.

Infantis strain, the *floR* and *tet(G)* genes as well as *orf1* and *orf2* (Fig. 1B), which can be lost together if recombination between the duplicated 5'-CS or 3'-CS regions occurs (3), were also missing, indicating loss of the central region.

Because seven of the nine strains were resistant to trimethoprim, additional screens for two trimethoprim resistance genes that have been detected previously in SGI1 variants (3, 10, 12) were also performed. These were for the *dfrA1* gene, which is found in a gene cassette (35), and the *dfrA10* gene (27), which is found associated with CR1 (32). The two Kiambu strains, which contain the five resistance genes of In104 but were additionally resistant to trimethoprim, also included the *dfrA10* gene (Table 3) and CR1. The serovar Infantis strain also included CR1 and the *dfrA10* gene. Four strains included the cassette-associated *dfrA1* gene either alone or together with either the cassette-associated *aadA2* or *blaP1* gene, suggesting that one or both of the cassette arrays had been exchanged.

Variation in the gene cassettes. The regions containing gene cassettes were amplified using standard primers in the 5'-CS and 3'-CS of class 1 integrons (21) (Fig. 1B). The DT104 control strain as well as the serovar Kiambu and serovar Paratyphi B strains yielded the 1.0- and 1.2-kb amplicons containing, respectively, the *aadA2* and *blaP1* cassettes, as expected for In104 (SGI1). However, the serovar Derby strain, which does not include the *blaP1* gene, gave an indistinguishable pattern (Fig. 2, top panel), indicating that the cassette amplicon sizes alone are insufficient to reliably indicate the presence of the original In104 configuration (Fig. 1).

Digestion of the PCR fragments with the restriction enzymes *RsaI* and *Tsp5091* was therefore used to distinguish and more accurately identify the cassette arrays. For the serovar Kiambu and serovar Paratyphi strains, where both the *aadA2* and *blaP1* cassette had been detected (Table 3), the restriction fragment patterns were identical to that of the MR DT104 (In104 type) control (Fig. 2, lower panel). However, the serovar Derby strain yielded a different pattern, confirming that it does not contain the *blaP1* cassette found in SGI1-containing DT104. The three strains that apparently yielded a single 1.2-kb PCR product (Fig. 2, top panel) were differentiated into two types, one (serovars Cerro and Dusseldorf) with two different cassette arrays and one (serovar Emek) with one array. The fragments seen in digests from the Emek strain were also seen in

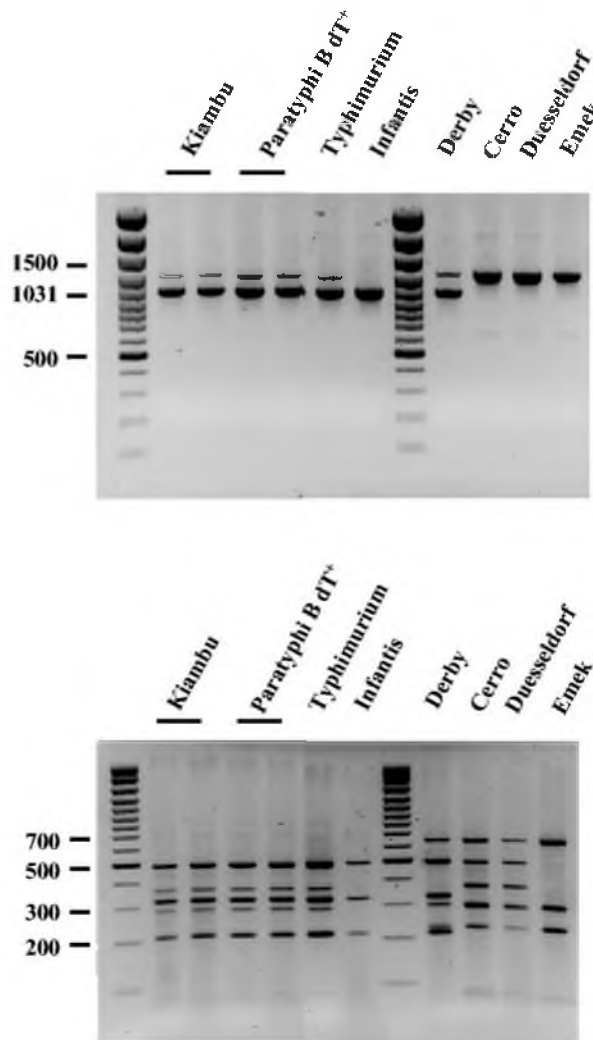


FIG. 2. Characterization of the cassette arrays. PCR amplification products (top panel) of cassette regions (using primers L1 and R1) and *RsaI*-generated restriction endonuclease fragments from them (bottom panel) were separated on agarose gels. Size markers are a 100-bp ladder.

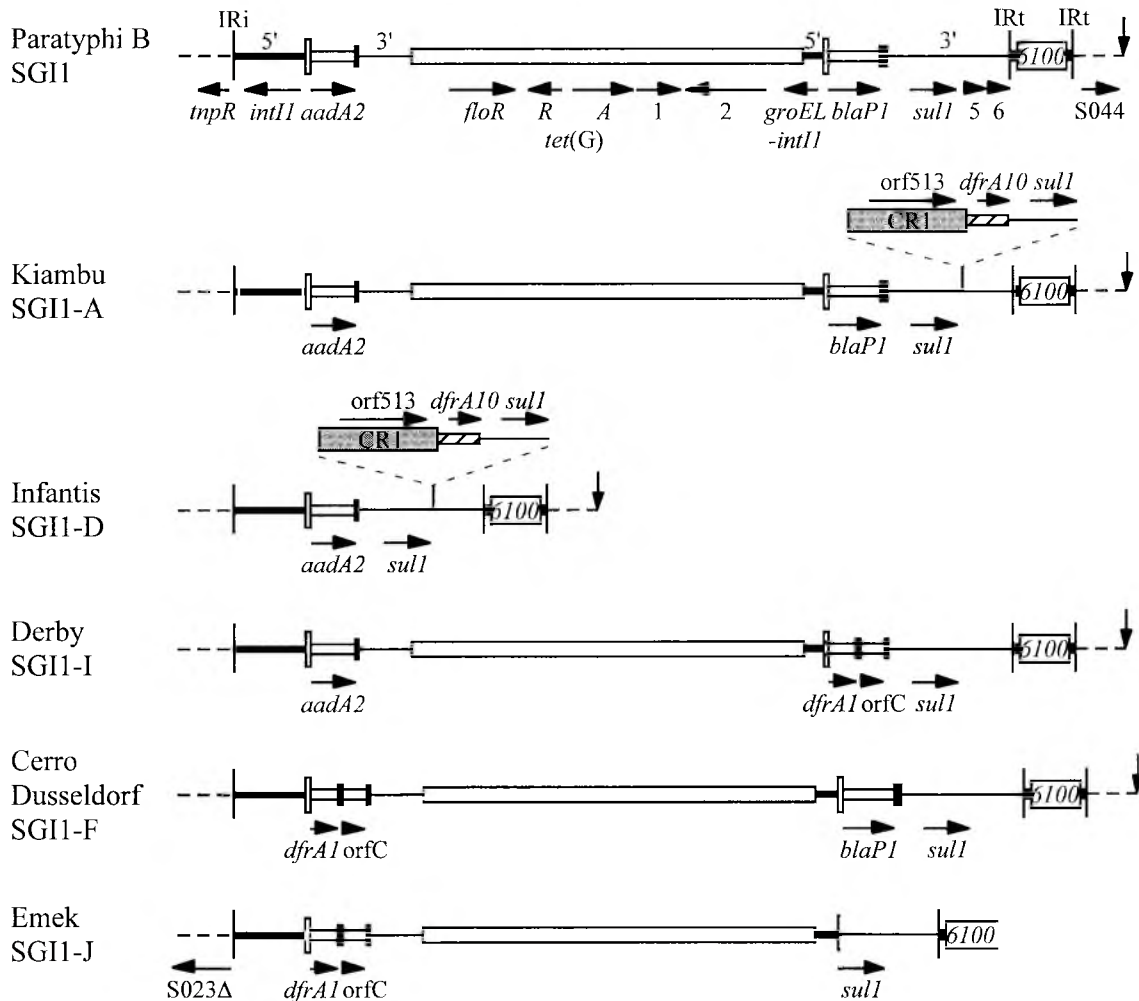


FIG. 3. Maps of In104 variants. Features are as in Fig. 1B, and the vertical arrow indicates the right-hand boundary of SGI1. Regions associated with the additional *dfrA10* genes are indicated above, with CR1 as a shaded box and the unique region including the *dfrA10* gene as a hatched box. The strain or strains from which each map is derived, together with the SGI1 designation, are indicated on the left.

the serovars Cerro, Derby, and Dusseldorf digests, where they are combined with bands corresponding to either the *aadA2* amplicon (seen alone in serovar Infantis) or the *blaP1* amplicon.

The Infantis strain yielded a single 1.0-kb product, consistent with the presence of only one *attI1* site containing the *aadA2* cassette in this strain, and this was confirmed by sequencing. To identify the cassette array in the isolates which contained the *dfrA1* gene, the single 1.2-kb product from serovar Emek was sequenced. It contained the *dfrA1* cassette with the orfC cassette and is identical to a cassette array reported in *Vibrio cholerae* (46) (GenBank accession no. AF455254), *Escherichia coli* (AB161449), and *Salmonella* spp. (AB186122). The restriction fragment analysis indicated that the same cassette array was also present in serovars Cerro, Derby, and Dusseldorf, and this was confirmed by PCR using a primer in each cassette. Hence, the Cerro and Dusseldorf strains include both the *dfrA1*-orfC and *blaP1* cassette arrays and serovar Derby includes *dfrA1*-orfC with *aadA2* (Fig. 3).

Location of the gene cassettes. As there is no a priori reason why the cassette arrays amplified could not have arisen from

integrons on resident plasmids rather than the integron in SGI1, further mapping was performed to confirm that the gene cassettes were part of the integron in SGI1 and to assign the cassettes to the left- or right-hand *attI1* site. This was achieved by PCR using one primer within the cassette and one in a region unique to the surroundings of the left- or right-hand *attI1* site (Fig. 1B). These were within S026 in the SGI1 backbone or in the *floR* gene for the left side and in the *groEL* region or orf5 for the right side (Table 1, Fig. 1B). The assignments are shown in Fig. 3. The serovar Emek strain was found to have the *dfrA1*-orfC cassette combination only on the left-hand side. A 1.2-kb *groEL*-*sul1* product was generated by amplification using the *groEL*-F and QS-2 primers (Table 1) and sequenced to determine the structure of the right-hand side. A deletion that removes both the gene cassette and 472 bp of adjacent sequences, including part of the *attI1* site and all of *qacEΔ1*, and is identical to a deletion found in Tn610 (23) (GenBank accession no. X53635) was present in this position.

Structure of the SGI1 integron variants. A complete map of each In104 homologue (Fig. 3) was obtained by establishing PCR linkage between most adjacent gene pairs using the for-

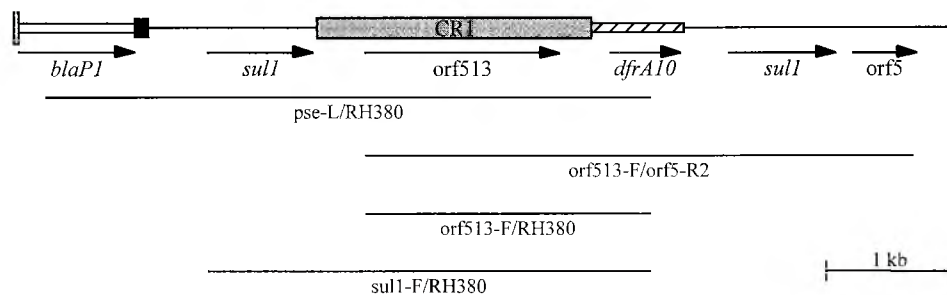


FIG. 4. Mapping the CR1-*dfrA10* region. The primer combinations used to link genes in serovar Kiambu and serovar Infantis strains that possessed CR1 are shown. Primer sequences are listed in Table 1.

ward primer of the first with the reverse primer of the second as well as longer linkages (Table 1). Linkage between *orf2* and *tetA*(G) was established by Southern hybridization with *tetA*(G) and CR3 digoxigenin-labeled probes to BsaI-digested whole-cell DNA. As predicted from the DNA sequence of SGI1 (4), a 4.1-kb BsaI fragment hybridized with the *tetA*(G) and *orf2* probes in all the SGI1-containing strains except serovar Infantis. The presence of the CR1-*dfrA10* region was confirmed, and its location was determined using PCR primers in the relevant cassettes with a primer in CR1 to link it to the right-hand or left-hand cassette array (Fig. 4). The integrons detected here include the original SGI1 (4) as well as the previously identified types designated SGI1-A, SGI1-D, and SGI1-F (3, 11, 12). In addition, two new types were found and designated SGI1-I (in serovar Derby) and SGI1-J (in serovar Emek) using the next available letters.

DISCUSSION

Since the discovery of genomic island SGI1 containing In104 in *S. enterica* DT104 strains, SGI1 or SGI1 homologues with an integron related to In104 have been found in several serovars of *S. enterica*. The range now includes serovar Typhimurium phage types DT104, DT120, DT12, DT1, and U302 (4, 6) and serovars Agona (3, 4, 7, 10), Paratyphi B dT⁺ (24, 25, 44; this study), Albany (12), Meleagridis (14), and Newport (13) as well as serovars Emek, Cerro, Derby, Dusseldorf, Infantis, and Kiambu identified in this study. This wide distribution provides a strong indication that the SGI1 genomic island is a significant determinant of multiple drug resistance in *Salmonella* and is clearly able to move from strain to strain.

One way in which this horizontal gene transfer could occur is by Int-mediated excision of SGI as a circular molecule, which is then packaged into a bacteriophage and transduced into another strain, and such transduction has been demonstrated (41). However, for each serovar, horizontal transfer need not necessarily have occurred on many occasions. The selective advantage conferred by the resistance genes in the many environments where antibiotics are used heavily, such as in the intensive rearing of food-producing animals, means that a single cell that had acquired SGI1 could be amplified (clonal expansion) and come to represent a significant fraction of the population of that serovar. The larger number of bacteria arising from such amplification would in turn increase the likelihood of spread of the new strain to new locations, and this is

clearly what has occurred in the case of SGI1-containing DT104 (36).

SGI1-A has been shown to be prevalent in poultry in Belgium (11), but whether any of the remaining serovars described here or by others are prevalent, particularly in food-producing animal populations, in the countries (including Australia) from which the isolates studied here originated remains to be established. The pairs of the same serovar isolates (Paratyphi B dT⁺ and Kiambu) identified here, though not known to be linked epidemiologically, could nonetheless be members of a single clonal line. In support of this, XbaI-digested chromosomal DNA from the Paratyphi B dT⁺ strains gave identical pulsed-field gel electrophoresis profiles (R. S. Levings and S. P. Djordjevic, unpublished observations). In addition, the properties of the multidrug resistance region in an *S. enterica* serovar Emek isolated in the United Kingdom (34) are consistent with its being within an SGI1 variant that is the same as SGI1-J in the serovar Emek strain studied here. Moreover, it appears that the absence of SGI1-containing DT104 in Australia does not reflect an absence of SGI1-containing strains belonging to other serovars.

Variation in the resistance gene content of the SGI1 integron observed here is likely to have arisen mostly by homologous recombination between identical segments, one within the integron in the SGI1 island and the second in a coresident plasmid or a smaller nonreplicating circular molecule. The latter can arise from a plasmid via homologous recombination as demonstrated for the CR-*dfrA10*-*sul1* circle (32). Gene exchange by homologous recombination is likely to represent an important route for the exchange of gene cassettes in class 1 integrons because they are flanked by two regions, the 5'-CS and the 3'-CS, that are present in most class 1 integrons (16, 17, 32). This mechanism has been demonstrated experimentally (30, 31) and is likely to be responsible for the substitution of the *dfrA1*-*orfC* cassette array for either the *aadA2* or the *blaP1* cassette in the isolates studied here and previously.

The deletion removing part of the *attI1* site found in one strain studied here (SGI1-J) could also have been acquired by this route from Tn610 (23) and, as Tn610 was originally found in *Mycobacterium fortuitum*, this suggests that this transposon has a very broad distribution. Previous claims of movement of gene cassettes from In104 into plasmids containing a class 1 integron via site-specific recombination (40) are unlikely to be correct. Cassette exchange most probably occurred by homologous recombination, granted that the SGI1-containing DT104

strains used are wild type and hence recombination proficient. Small nonreplicating circular molecules that have arisen by homologous recombination from a class 1 integron that has direct duplication of either the 5'-CS or 3'-CS, such as those found in In104 and those in CR1(orf513)-containing integrons (32, 42), are also likely to be important. These circles, once formed, can recombine into new locations, e.g., plasmids that contain a copy of any sequence included within them. The *dfrA10* gene has been shown experimentally to move in this way (32) and is likely to have entered the In104 variants that contain it via this route. This route is also one that could lead to the movement of the *floR-tet(G)* region onto a plasmid that contains an integron and thence out into the broader population of *Enterobacteriaceae*. Indeed, such a plasmid may represent the original source of these genes in In104.

A particularly surprising finding of this study was that SGI1 or an SGI1 variant was found in 8 of the 39 strains included in the study that were isolated from humans with severe *Salmonella* infections. Recently, a single case of a human infection with SGI1-containing serovar Agona (11) and sporadic cases of serovar Paratyphi B dT⁺ infections in humans in Canada and the United Kingdom (25, 44) have also been reported. The United Kingdom Paratyphi B strains are associated with owners of tropical fish tanks, as are the Australian isolates reported here. As these serotypes are historically viewed as having low infectivity for humans, it was suggested that the presence of SGI1 may increase the likelihood of human infection (25). Our findings also support the contention that the presence of SGI1 introduces a determinant that may predispose to human infection. However, the possibility that these human infections simply reflect a widespread distribution of SGI1-containing *Salmonella* serovars in animal populations in the Southeast Asia-Australia region and in poultry in Belgium is still to be eliminated.

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